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1. Your reference IGH 0003

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2. Patent application number
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3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

Dr Camilo Colaco
107 Foster Road
Cambridge CB2 2JN
United Kingdom

Patents ADP number *(if you know it)* 6814107001

If the applicant is a corporate body, give the country/state of its incorporation

4. Title of the invention
Trehalose producing cells as vaccines

5. Name of your agent *(if you have one)*

Dr Camilo Colaco

Address for service in the United Kingdom to which all correspondence should be sent *(including the postcode)*

107 Foster Road
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Patents ADP number *(if you know it)*

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Description 7

Claim(s) 1 (10 claims)

Abstract 1

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Statement of inventorship and right to grant of a patent (Patents Form 7/77)

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12 Name and daytime telephone number of person to contact in the United Kingdom

Dr Camilo Colaco Tel. 01223 841870

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Docket No: IGH 0003

TITLE: TREHALOSE PRODUCING CELLS AS VACCINES

This invention relates to the field of vaccines. More specifically, it relates to methods of producing vaccines of trehalose containing procaryotic cells and the compositions obtained thereby.

BACKGROUND ART

Procaryotic cells, particularly bacteria, are widely and increasingly used in important medical, agricultural and industrial applications. Agricultural or environmental, applications include biopesticides and bioremediation. Medical applications include use of bacteria in vaccines as well as for production of pharmaceutical products for treatment and numerous industrial compositions. The use of bacterial vaccines promises only to increase, given the dramatic rise in biotechnology as well as the intensive research into the treatment of infectious diseases over the past twenty years.

For these cells to be used effectively both in terms of desired results and cost, the cells must be able to be stored for significant periods of time while preserving their viability. This has proven to be a major difficulty. Methods for preserving live procaryotic cells suffer from several serious drawbacks, such as being energy-intensive and requiring cold storage. Thus freeze-drying is often used for preservation and storage of procaryotic cells. However, it has undesirable characteristics of significantly reducing viability as well as being time- and energy-intensive and thus expensive. PCT GB97/03375 describes a process of stabilising procaryotic cells by the induction of trehalose synthesis and the drying of the resulting cells in a glassy carbohydrate matrix. This process gives stabilised cells that can be stored at ambient temperatures without loss of viability. Trehalose, (α-D-glucopyranosyl-α-D-glucopyranoside), is a naturally occurring, non-reducing disaccharide which was initially found to be associated with the prevention of desiccation damage in certain plants and animals which can dry out without damage and can revive when rehydrated. Trehalose has been shown to be useful in preventing denaturation of proteins, enzymes and foodstuffs during desiccation. See U.S. Patent Nos. 4,891,319; 5,149,653; 5,026,566. Colaco et al. (1992) *Bio Tech.* 10:1007-1011. Trehalose synthesis is induced by a number of methods including osmotic shock which induces the endogenous production of trehalose. Welsh et al. (1991) *J. Gen. Microbiol.* 137:745-750.

PCT application No. GB94/01556 describes a process of improving the viability of bacterial dried cells by the induction of trehalose synthesis by nutrient limitation, heat shock or osmoadaptation. PCT application No. GB97/03375 describes a method for the preservation of procaryotic cells by the drying of cells in a carbohydrate matrix after the induction of trehalose

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hesis. The latter invention provides compositions of dried cells that can be stored at ambient temperatures and thus enable a number of industrial applications.

In the process of testing the dried stabilised procaryotic cells as vaccines, we have now found that they were notably much more antigenic than fresh live controls. Suprisingly, we find that this increased immunogenicity of the stabilised procaryotic cells is not dependent on the drying process but results from the induction of trehalose synthesis; though more notable with dried cells, this increased immunogenicity is also seen in cells induced to produce trehalose but not dried prior to use as a vaccine.

SUMMARY OF THE INVENTION

The present invention encompasses methods of producing vaccines of procaryotic cells induced to synthesise trehalose. The invention also includes methods for the induction of trehalose synthesis. The invention further includes the drying of the induced cells prior to use as a vaccine. The invention also includes compositions produced by these methods.

As used herein, "procaryotic cells" are cells that exhibit characteristics of prokaryotes, which is a term well known in the art. Prokaryotes are typically unicellular organisms and lack organelles (such as mitochondria, chloroplasts, and Golgi apparatus), a cytoskeleton, and a discrete nucleus. Examples of procaryotic cells include bacteria, such as eubacteria, cyanobacteria and prochlorophytes; archaebacteria; and other microorganisms such as rickettsias, mycoplasmas, spiroplasmas, and chlamydiae. For purposes of this invention, prokaryotes are capable of synthesizing trehalose. This ability can be native or conferred by recombinant techniques. The ability to synthesize trehalose can be determined by measuring trehalose concentration, which is described below. Preferably, the procaryotic cells are bacteria.

Conditions that "increase trehalose concentration" are conditions that initiate, encourage, allow, and/or increase the rate of synthesis of trehalose within the cell(s), and/or increase the amount of trehalose within the cell(s) when compared to growing the cell(s) without these conditions. Conditions (including preferred conditions) which stimulate production of intracellular production of trehalose are discussed in detail below. Examples of these conditions include, but are not limited to, growing the cell(s) under stressful conditions such as osmotic shock, i.e., high salt conditions, nutrient limitation and heat shock (see eg. PCT application Nos. GB94/01556 and GB97/03375). Conditions that stimulate production of trehalose may also be effected by, for example, inhibiting the rate of degradation of trehalose.

"High osmolarity" refers to excessive solute concentration in growth media. "Excessive" solute concentration means that solute concentration (generally salts) is above the level at which a cell exists and/or grows in its native environment.

"Viability" is a term well understood in the art, and is consonantly used herein to mean manifestations of a functioning living organism, such as metabolism and cell division. Methods to measure viability are known in the art and are described herein.

The present invention encompasses methods of inducing trehalose synthesis in procaryotic cells and the cells produced thereby. These methods comprise the steps of culturing the procaryotic cells under conditions that increase trehalose concentration and using these induced cells as vaccines. The cells may be dried in the presence of a non-reducing carbohydrate for storage prior to use as a vaccine.

Growing procaryotic cells to stimulate trehalose synthesis. To practice the methods of this invention, procaryotic cells are grown under conditions that increase trehalose concentration. Trehalose can be measured using standard methods in the art. Any procaryotic cell, particularly bacteria, containing trehalose synthase genes should be capable of producing trehalose. Many types of procaryotic cells are known to synthesize trehalose. Bacteria have two genes involved in trehalose synthesis (i.e. T-Phosphate synthase and T-P phosphatase), whereas yeast have at least three genes and combinations of these genes have been used to enable trehalose synthesis. Examples of bacteria that contain the trehalose synthase gene include, but are not limited to, Enterobacteriaceae, such as *Salmonella* and *Escherichia (e.g., S. typhimurium* and *E. coli)*; halophilic and halotolerant bacteria, such as *Ectothriorhodospira (e.g., E. halochloris);* micrococcocaceae, such as *Micrococcus (e.g., M. luteus)*; *Rhizobium* species such as *R. japonicum and R. leguminosarum bv phaseoli*; *Cyanobacteria*; *Mycobacteria* species such as *M. tuberculosis, M. bovis,* and *M. smegmatis.*

Procaryotic cells can be induced to produce trehalose by culturing the cells under stressful conditions, e.g., osmotic shock, heat or oxygen limitation (shock), carbon/nitrogen starvation, or any combination of the above. Alternatively, use of inhibitors of enzyme(s) involved in trehalose degradation (i.e., trehalase), such as validomycin, may also result in accumulation of trehalose. Suitable conditions can be determined empirically and are well within the skill of one in the art. While not wishing to be bound to a particular theory, induction of trehalose production under stressful conditions may trigger synthesis or accumulation of other molecules that may be beneficial for preservation, such as betaine and chaperonins.

For bacteria, particularly *Escherichia,* trehalose production can be stimulated by growing the cell(s) in conditions of high osmolarity, i.e., salt concentrations sufficient to stimulate trehalose production. Thus, the invention includes culturing procaryotic cells in osmolarity of at least about 350 mOsmoles to about 1.5 Osmoles, preferably at least about 400 mOsmoles to 1 Osmole. More preferably 250 mOsmoles to 500 mOsmoles. Generally, a

mum salt concentration of about 200 mOsmoles is required. A single salt can be sufficient to stimulate trehalose production, for example, 200 mM NaCl. KCl and $CaCl_2$ also stimulate trehalose synthesis, indicating that trehalose synthesis is not dependent on the action used or the concentration of chloride in the growth medium. When $(NH_4)_2SO_4$ is used, however, only about one half of the amount of trehalose is produced compared to that produced in the presence of KCl, NaCl and $CaCl_2$. A combination of salts can also be used. In addition, when used to increase the osmolarity of the medium, a non-penetrant solute such as sorbitol and/or glucose can contribute to the stimulation of trehalose accumulation.

The salt concentration (i.e., osmolarity) required to stimulate and/or induce trehalose production will depend upon the genus, species, and/or strain of the procaryotic cell used. Preferably, cell(s) are grown in a minimal medium containing salt. Commercially available minimal medium is supplemented with desired salts and/or other solutes may be used, although minimal medium is not essential and defined media can also be used. The time required to initiate and achieve the desired level of trehalose concentration will vary depending on the level of osmolarity as well as the genus, species and/or strain of procaryotic cell used and can be determined empirically. Trehalose synthesis will generally begin within an hour of placing cells in condition designed to stimulate trehalose production. Generally, in *E. coli* the amount of trehalose reaches a maximum at about 15-20 hours after placing cells in conditions that stimulate trehalose production.

To induce trehalose synthesis by osmotic shock, the total concentration of salt(s) in the medium should be at least about 0.2 M, preferably at least about 0.4 M, more preferably at least about 0.5 M. The total concentration of salt(s) should not exceed 0.6 M. At about 0.6 M or above, trehalose synthesis declines in *E. coli*. The salt concentration required for the desired result may vary depending on the general/species/strain used, and can be determined empirically.

Production of trehalose may also be stimulated using recombinant methods which are well known in the art. For instance, procaryotic cells can be transfected with a DNA plasmid comprising a DNA sequence encoding the appropriate trehalose synthase gene. The gene in turn is operatively linked to a suitable promoter, which can be constitutive or inducible. Recombinant methods are described in a variety of references, such as "Molecular Cloning: A Laboratory Manual," second edition (Sambrook *et al.*, 1989).

Trehalose can be measured by using assays known in the art, such as by high pressure liquid chromotography (HPLC), coupled with electro-chemical detection and glucose assay (Trinder assay using trehalase) for quantitative enzymatic determination of trehalose. Thin layer

chromatography can be used as a qualitative method for the separation of different carbohydrates. Refractive index detection provides another means of detecting sugars quantitatively.

In measuring trehalose by HPLC, cells are disrupted and trehalose preferentially solubilized in 70% ethanol, followed by removing triglycerides by chloroform extraction. Trehalose concentration is determined by multiplying trehalose concentration (as determined by a standard curve) by the fraction of final volume of supernatant divided by pellet volume. A more detailed description of this assay is provided in Example 1.

Preferably, the concentration of trehalose is at least about 10 mM; more preferably, at least about 30 mM; more preferably, at least about 50 mM; and even more preferably, at least about 100 mM. Thus, the invention includes culturing the procaryotic cells under conditions that stimulate intracellular production of trehalose, wherein intracellular concentration of trehalose reaches at least about 10 mM, preferably at least about 30 mM, more preferably at least about 50 mM, more preferably at least about 100 mM, and even more preferably at least about 150 mM.

The time required for stimulating trehalose synthesis depends, inter alia, on the nature of the procaryotic cells (including genus, species, and/or strain) and the conditions under which trehalose induction occurs (i.e., whether by osmotic shock, oxygen deprivation, etc.). For trehalose induction by osmotic shock, the time required for maximum concentration of trehalose in turn depends on the degree of osmolarity as well as the particular salts used. For example, in *E. coli*, ammonium sulfate ($(NH_4)_2SO_4$) stimulates about half the amount of trehalose concentration as NaCl, $CaCl_2$ or KCl. For *E. coli* in 0.5 M salt minimal media, maximum trehalose concentration occurs within about 10–17 hours, with significant induction by 17 hours after osmotic shock .

The induced cells may then be frozen for storage before use as a vaccine. Alternatively storage of the vaccine can be effected by culturing the procaryotic cells under conditions that increase trehalose concentration to a level effective to increase storage stability, mixing the cells with a drying solution which contains a stabilizing agent, and drying the cells under conditions such that a glass is produced having less than about 5% residual moisture. If a killed vaccine rather than a live vaccine is required the cells may be killed by any method known in the art including chemical fixation and radiation prior to processing for storage. Though the procaryotic cells may be used as sole components of the vaccine, an adjuvant may be added in an amount sufficient to enhance the immune response to the procaryotic vaccine. The adjuvant can be added to the procaryotic cells before drying, for example, cholera B toxin subunit can be

dried simultaneously with *V. cholera*. Alternatively the adjuvant may be obtained and dried separately, and reconstituted along with the procaryotic cells.

Suitable adjuvants include, but are not limited to, aluminum hydroxide, alum, QS-21 (U.S. Pat. No. 5,057,540), DHEA (U.S. Pat. Nos. 5,407,684 and 5,077,284) and its derivatives (including salts) and precursors (e.g., DHEA-S), beta-2 microglobulin (WO 91/16924), muramyl dipeptides, muramyl tripeptides (U.S. Pat. No. 5,171,568), monophosphoryl lipid A (U.S. Pat. No. 4,436,728; WO 92/16231) and its derivatives (e.g., Detox™), and BCG (U.S. Pat. No. 4,726,947). Other suitable adjuvants include, but are not limited to, aluminum salts, squalene mixtures (SAF-1), muramyl peptide, saponin derivatives, mycobacterium wall preparations, mycolic acid derivatives, nonionic block copolymer surfactants, Quil A, cholera toxin B subunit, polyphosphazene and derivatives, and immunostimulating complexes (ISCOMs) such as those described by Takahashi et al. (1990) *Nature* 344:873-875. The choice of an adjuvant will depend in part on the stability of the vaccine in the presence of the adjuvant, the route of administration, and the regulatory acceptability of the adjuvant, particularly when intended for human use. For instance, alum is approved by the United States FDA for use as an adjuvant in humans.

Compositions of cells made by the methods herein. The invention also encompasses compositions comprising procaryotic cells obtained by the methods described herein. The compositions include, but are not limited to, vaccines comprising induced procaryotic cells and dried procaryotic cells made according to the methods described herein. The compositions may further comprise any adjuvant, pharmaceutically acceptable vehicle or excipient, which are well known in the art.

The following examples are provided to illustrate but not limit the invention.

EXAMPLES

Example 1: Induction of trehalose in *E. coli* by osmotic shock

E. coli NCIMB strain 9484 was cultured in Evans medium (pH 7.0; Table 1). After overnight incubation at 37°C in initial Evans medium a 4 ml culture of *E. coli* grown in Evans medium under nitrogen limitation was used to inoculate a 200 ml culture of Evans medium modified to induce osmotic shock by increasing the salt concentration (KCl) to 0.5M.

Trehalose concentration was measured by high pressure liquid chromatography (HPLC) analysis and significant increases in trehalose concentrations were observed at 15–17 hours after initiation of osmotic shock, with values peaking at less than 20 hours.

Example 2: Induction of trehalose synthesis in *Salmonella*

Salmonella typhimurium (1344) was grown overnight at 37°C in either M9 (minimal) medium with and without 0.5 M NaCl. Cells were harvested by centrifugation and analyzed for trehalose concentration by HPLC analysis as described in Example 1. Growth in high salt medium showed at 4 to 5 fold induction of trehalose synthesis.

Example 3: Drying of procaryotic cells after induction of trehalose synthesis

E.Coi and *Salmonella typhimurium* was grown overnight at 37°C in either M9 (minimal) medium with and without 0.5 M NaCl and trehalose synthesis induced as described in examples 1 and 2. The induced bacteria were harvested by centrifugation at 10,000 rpm for 10 minutes and the cell pellets resuspended in drying solution containing 45% trehalose, 0.1% cmc (sodium carboxymethyl cellulose; Blanose 7HF; Aqualon) to a typical cell density of 0.5-1.2 x 10^9 CFU/ml. 300µl and 500µl aliquots were dispensed into 3ml pharmaceutical vials and dried under vacuum without freezing, overnight at ambient temperature and a vacuum pressure of 30mTorr or freeze-dried using the following protocol: ramp at 2.5°C/min to an initial shelf temperature of -40°C; primary drying was performed at a vacuum pressure of 30mT at -40°C and hold for 40 hours; for secondary drying ramp at 0.05°C/min from -40 to 30°C and hold for 12 hours.

Example 4: Use of induced procaryotic cells as vaccines

E.Coli and *Salmonella typhimurium* induced to synthesise trehalose as in Examples 1 and 2 were used to immunise mice and rabbits. Titration of the bacteria showed that a 100 to 1000 fold lower titre of bacteria induced for trehalose synthesis was required to produce an equivalent antibody response in the animals immunised with uninduced bacteria. Dried preparations were generally 2-50 fold more effective on a cell number basis at eliciting protective immunity in the immunised animals.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be apparent to those skilled in the art that certain changes and modifications may be practiced. Therefore, the description and examples should not be construed as limiting the scope of the invention, which is delineated by the appended claims.

CLAIMS

We claim:

1. A method of producing a vaccine of procaryotic cells comprising the steps of:
   a) culturing the procaryotic cells under conditions that increase trehalose concentration to at least 10mM;
   b) using the procaryotic cells obtained in step to immunise an animal species

2. The method according to claim 1, wherein the culturing is in osmolarity sufficient to increase trehalose synthesis.

3. The method according to claim 2, wherein the osmolarity is at least about 350 mOsmoles -1.5 Osmoles

4. The method according to claim 1, wherein the culturing is at a temperature sufficient to increase trehalose synthesis.

5. The method according to claim 1, wherein the procaryotic cells are bacteria.

6. The method according to claim 1, wherein the procaryotic cells are protozoa.

7. The method according to claim 1, wherein the procaryotic cells are fungi.

8. The method according to claim 1, wherein the cells are dried prior to use as a vaccine.

9. The method according to claim 1, wherein the cells are killed prior to use as a vaccine.

10. A composition obtained according to the method of any of the above claims.

ABSTRACT

This invention provides methods of using procaryotic cells induced to synthesise trehalose as vaccines, and the compositions obtained thereby.